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**CHARACTERIZATION OF THE SELENIUM**

**COMPOUNDS IN RAT BILE**

This thesis is submitted as a creditable and  
independent investigation in accordance with the degree  
Master of Science and is acceptable for meeting the  
thesis requirements of a committee. Acceptance of this  
thesis does not imply any endorsement or approval by  
the candidate or the committee of the conclusions of the  
work presented.

by

**JAMES TERRENCE BAUMBERGER**

A thesis submitted  
in partial fulfillment of the requirements for the  
degree Master of Science  
Major in Chemistry  
South Dakota State University  
1986

## CHARACTERIZATION OF THE SELENIUM

### COMPOUNDS IN RAT BILE

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Dr. Ivan S. Palmer  
Thesis Advisor

Date

Dr. David C. Hilderbrand  
Head, Chemistry Department

Date

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## INTRODUCTION

Selenium, atomic number 34, resembles sulfur in many of its chemical properties and occurs in inorganic form as  $\text{H}_2\text{Se}$ ,  $\text{H}_2\text{Se}_2\text{O}_3$ ,  $\text{H}_2\text{SeO}_3$ , and  $\text{H}_2\text{SeO}_4$  which are the analogues of  $\text{H}_2\text{S}$ ,  $\text{H}_2\text{S}_2\text{O}_3$ ,  $\text{H}_2\text{SO}_3$ , and  $\text{H}_2\text{SO}_4$  respectively. It is located between sulfur and tellurium in the Periodic Table and exists in the same valency states as sulfur, namely, -2, 0, +2, +4, and +6.

In contrast to sulfur oxides, the most stable oxide of selenium is  $\text{SeO}_2$  rather than  $\text{SeO}_3$ . Selenium dioxide is a solid that readily reacts with water to form  $\text{H}_2\text{SeO}_3$  and is easily reduced to elemental selenium.

Besides inorganic forms, numerous organic selenium compounds are known. Organoselenium compounds, in general, are less stable and more reactive than the corresponding sulfur analogues and these properties may account for the toxicity of selenium when it is incorporated in place of sulfur in cellular constituents.

Extensive investigations in connection with a cattle ailment known as "alkali disease", eventually showed it to be connected with the selenium content of the soil. Research starting in the 1930's demonstrated that certain plants were capable of incorporating and accumulating selenium, and that these plants were toxic

to animals. Since these discoveries, many efforts have been made to find suitable means of control and protection against selenium toxicity.

In 1938, Moxon discovered the first protective factor; demonstrating the ability of arsenic to protect against selenium toxicity (1). From this original observation, a wealth of information has been generated including the demonstration that arsenic exerts its effect by increasing the biliary excretion of selenium. To date, the form of selenium compounds in bile has not been characterized.

The studies reported here are an attempt to investigate more thoroughly the forms of selenium compounds excreted into bile. Since preliminary studies indicated the possibility of selenium excretion into bile associated with protein, the study concentrated in this area. Other compounds such as phospholipids, lipids, cholesterol, and bile salts were also investigated. Finally, smaller metabolites, including selenoamino acids and glutathione were studied for their possible association with selenium.

## LITERATURE REVIEW

Selenium is an element which can be taken up by plants and in sufficient quantity, will cause toxic signs in animals. The toxicity of the element is the subject of several reviews (2,3,4), however, nutritional research over the years has shown that selenium is also an essential nutrient required for the prevention of a number of serious deficiency diseases in various species of livestock and poultry. This unexpected nutritional role of selenium emerged in 1957 with the discovery by Schwarz and Foltz (5) that the element was the unknown factor, previously found in yeast, kidney, and liver, required for prevention of dietary necrosis in vitamin E-deficient rats.

Since 1957, inorganic selenium compounds, such as sodium selenite or sodium selenate, have been shown to be intimately involved, together with vitamin E, in the prevention of a wide variety of nutritional deficiency diseases. Additional proof of the specific nutritional essentiality of selenium was obtained by Thompson and Scott (6) who showed that chicks and Japanese quail, receiving diets containing synthetic amino acids and other purified ingredients extremely low in selenium, failed to grow and suffered high mortality

unless their diet was supplemented with selenium. Addition of all known nutrients, including high levels of vitamin E, failed to prevent severe deficiency signs and death, whereas addition to the basal high vitamin-E diet of as little as 0.02 ppm selenium (as sodium selenite) completely prevented all deficiency signs and promoted a good rate of growth in the chicks and quail. Subsequent studies have shown that selenocystathionine, selenomethionine, and selenocystine all are approximately as effective as selenium from selenite in preventing dietary liver necrosis and other deficiency diseases.

Before the recognition of the deficiency diseases, selenium was known to be a very toxic substance. This knowledge hindered research on effects of trace levels of the element since selenium as an essential nutrient was just not accepted. Therefore, research centered on the toxic aspects of selenium rather than the therapeutic properties.

Japha proved as early as 1842 that selenium was toxic (7). It was associated with general livestock poisoning (alkali disease) in 1931 by Franke and coworkers (2). They concluded that soils, which had been derived from certain geological formations, contained relatively high levels of selenium. Plants

growing on these soils could absorb the selenium, concentrating it, and becoming toxic to the animals.

Since the discovery of the toxicity of selenium, several investigators have studied the metabolism of this element in the body and the possible means to protect against its toxicity.

#### SELENIUM METABOLISM:

Identified metabolites: Most studies on the metabolism of selenium in the body have been performed using the rat. In 1940, Schultz and Lewis (8) reported the lungs as a main excretory pathway of selenium in the rat. They found that 17-52% of a single dose of subcutaneously injected selenium, as sodium selenite, was excreted within 8 hours as volatile compounds in the respiratory gases. Later in 1952, McConnell and Portman (9) published substantial proof that dimethyl selenide was one of these volatile products exhaled in large amounts by rats injected with high levels of sodium selenite.

In 1969, Palmer et al. (10) isolated a major selenium metabolite from the urine of rats injected with  $^{75}\text{Se}$ -selenite. The metabolite was identified as a trimethylselenonium ion in agreement with the results of Byard (11). Thirty to fifty per cent of urinary selenium, in a 24 hour sample, was excreted as trimethylse-

lenonium ion after a single injection of selenite. Palmer et al. (10) suggested that it was a normal excretory product of selenite since it was excreted at both high and low levels of selenium when the selenite was administered either orally or by injection. Later it was shown that trimethylselenonium ion was a major excretory product not only from selenite but also selenate, selenomethioine, selenocystine, Se-methylselenocysteine, and seleniferous wheat (12).

Regarding other urinary metabolites, several laboratories (12,13) have reported the presence of a second metabolite (called U-2) as a rather diffuse spot near trimethylselenonium ion in paper chromatograms. U-2 is still unidentified.

Besides its excretion in the urine, selenium was also known to be excreted into bile. Initially, studies showed only 1.3-2.1% of injected selenate appeared in bile of dogs after 7 hours (14); however, later studies showed that this level could be greatly increased by simultaneous administration of arsenic (15).

Effects of arsenic on selenium metabolism: In 1938, Moxon (1) studied the toxicity of selenium in combination with tellurium, arsenic, vanadium, nickel, tungsten, and molybdenum. He discovered that the addition of arsenic ( $\text{NaAsO}_2$ ) to the drinking water gave full

protection against liver damage in rats caused by 15 ppm of selenium in the form of seleniferous wheat. This subject has drawn much subsequent attention, but will not be reviewed in this study.

In 1957, Palmer and Bonhorst (16) found that arsenic definitely delayed the entry of selenite into the liver with an accompanying high blood selenium level. They speculated that, since arsenite prevented exhalation of volatile selenium, it was reasonable to expect an increase in excretion by other routes; for instance urinary excretion of selenium might be increased. However, Ganther and Baumann (17) found that when selenium levels of 1.5 or 2.0 mg/kg of body weight were injected, urinary excretion of selenium was not affected by arsenite. However, arsenite did markedly increase the excretion of selenium into the gastrointestinal tract from 5-15% of the injected dose in the controls to 30-40% in the arsenic treated group. Kidney levels were also increased, while levels of selenium in the blood, liver, carcass, and the expired air were decreased by arsenite.

In 1966, Levander and Baumann (15) confirmed the increased elimination of selenium into the gut, in the presence of arsenic, under a variety of conditions including varied doses, forms of arsenic, and time



intervals between the injections. They also demonstrated that selenite stimulated the gastrointestinal excretion of arsenic, suggesting the possibility of a detoxification conjugate involving both elements. Finally, Levander and Baumann (18) showed that the increased gastrointestinal excretion of selenium in the presence of arsenic could be accounted for by increased biliary excretion. The effect of arsenic on the biliary excretion of selenium has been demonstrated with several forms of selenium and arsenic, but sodium arsenite was by far the most active form of arsenic tested compared to arsenate and organic arsenicals (18).

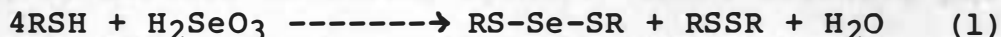
Since the discovery that arsenic increases the biliary excretion of selenium, very few characterization studies have been performed on the selenium compounds in bile. Preliminary studies by Andraos (19) in her Masters thesis suggested that selenium was possibly incorporated into proteins. To date, no selenium compounds have been identified in bile. Although studies on the form of selenium in bile are lacking, research has been directed toward other areas of the body.

Selenium has been found to concentrate in the protein fractions of a variety of organs including the muscles, liver, kidney, testes, and others. When selenium was administered in the diet as  $^{75}\text{Se}$ -selenite or



$^{75}\text{Se}$ -selenomethionine, association of the label with the protein fraction was noticed. Since this has been shown with a variety of other organoselenium compounds, a non-specific association of selenium with the thiol moiety of the proteins may be occurring.

Role of thiols: The reaction of selenite with thiol groups in cysteine residues, glutathione, or coenzyme A to form selenotrisulfides has been shown to be an important pathway by which inorganic selenium can be initially incorporated into living systems (20). Ganther has concluded that the usual combining ratio of thiol to selenite is 4:1 and that the stoichiometry of the reaction is as shown in equation 1.



When the thiol:selenious acid ratio exceeds 4:1, which is the case in biological systems, there is the possibility for reduction of the selenotrisulfide to the selenopersulfide:



Analogous reactions occur readily with trisulfides and would be expected to occur even more easily with selenotrisulfides because of the greater ease with which a S-Se bond is cleaved compared to an S-S bond.

Besides the selenotrisulfide and selenopersulfide derivatives of glutathione, a third compound yet to be identified was shown to be produced (21). It is possible that still other derivatives of glutathione may be formed under other conditions.

In rats, controversy has existed concerning the relative contribution of oxidized (GSSG) and reduced (GSH) forms of glutathione to the total glutathione efflux from the liver into bile. Recently, Eberle, et al. (22) found that bile, unlike liver cytosol or an in vitro buffer, had the unique ability to oxidize GSH rapidly ( $t_{1/2} = 5$  min) to the disulfide form by a nonenzymatic,  $O_2$ , pH-dependent chemical reaction inhibited only by certain chelating agents. Thus, selenite present in bile would most likely be reduced by the simultaneous oxidation of glutathione forming the known selenotrisulfide and selenopersulfide derivatives.

Besides the simple chemical reaction of selenium with glutathione, certain enzymes have been discovered to contain an essential selenium moiety.

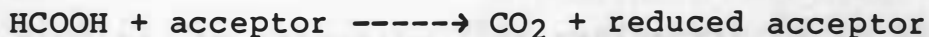
#### SELENIUM IN ENZYMES:

Almost 30 years ago Pincent (23) reported that selenium was essential for the expression of a specific enzyme activity in *E. Coli*. At that time, nutritionists and physiologists were aware only of the

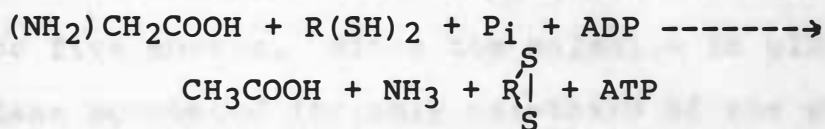
toxic effects of this element, thus general acceptance of these findings was delayed. Our present knowledge of the specific biochemical roles of selenium is very limited and the literature dealing with detailed studies at the enzyme level dates only from 1972. Now a total of five selenoenzymes are known to occur in bacteria (23-26). They are listed, together with the reactions they catalyze, below. The first selenoenzymes that were identified all proved to participate in oxidation-reduction reactions but the latest addition to the list, thiolase, is not a redox catalyst.

#### Redox Reactions

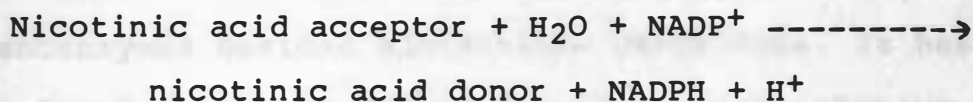
##### 1. Formate dehydrogenase (24, 25):



##### 2. Glycine reductase (25, 26):



##### 3. Nicotinic acid hydroxylase (27):



##### 4. Xanthine dehydrogenase (27):



#### Carbon-Carbon Bond Formation

##### 5. Thiolase (27):



Although many bacterial enzymes contain essential selenium, glutathione peroxidase has been the only mammalian enzyme shown to require selenium. Glutathione peroxidase is a selenoenzyme (28,33,34) that catalyzes the reduction of various hydroperoxides (29,30) as well as hydrogen peroxide (31) with glutathione as hydrogen donor. This enzyme has been suggested to function in animals by protecting the cellular membrane system against peroxidative damage (32). The importance of selenium as an essential trace element is largely concerned with this suggested function of the enzyme.

Recently, glutathione peroxidase along with other selenium proteins from selected tissues of the rat were studied by measuring  $^{75}\text{Se}$  radioactivity in animals fed  $^{75}\text{Se}$ -selenite as the main dietary source of selenium (35) for five months. Since the selenium in glutathione peroxidase accounted for only one-third of the whole-body selenium, there may be other important mammalian selenoenzymes besides glutathione peroxidase. It has been found that over 80% of the whole-body selenium was associated with protein and was distributed among seven subunit sizes and nine DEAE Sephacel chromatographic forms (36,43,44). Since most of these proteins did not have glutathione peroxidase activity, other selenoproteins must be present in rat tissues.

A number of reports suggest that a selenium-transport protein might exist in blood plasma(37-39), and another suggests that this selenoprotein is synthesized in rat liver and transfers selenium from the liver to extrahepatic tissues (40). Additional evidence suggests that selenoproteins other than glutathione peroxidase exist in kidney (41), liver (41), testes (42), and sperm (42).

#### FORMS OF SELENIUM IN PROTEINS:

The form of selenium in the various selenoproteins has been concluded to be selenocysteine; in fact, selenocysteine is the only form of selenium that has been reported in animals (45,46). Plants and bacteria contain other forms of selenium. How selenium becomes incorporated into these proteins has been the subject of much debate. Two hypotheses have been proposed.

The first hypothesis assumes that selenium, possibly in an inorganic form, is incorporated post-translationally, by a yet unknown reaction with an amino acid residue present in the backbone of the pre-formed protein. This hypothesis has been supported by the apparent inability of animals to synthesize free selenomethionine and the probable inability to produce free selenocysteine in a significant quantity from inorganic selenium compounds which are known to provide selenium

for glutathione peroxidase (45).

The second hypothesis assumes the existence of a specific codon for a rarely occurring amino acid selenocysteine. In support of this latter hypothesis, Hawkes et al. (47,48) suggested that selenocysteine was incorporated directly into the peptide backbone of the enzyme via a specific selenocysteine-tRNA. Evidence for isolation of this tRNA has been presented.

Whether selenium is present in glutathione, protein, a specific enzyme, or some other compound in bile remains to be seen. Several selenium-containing compounds are probably present.

The objectives of this study were to isolate and identify those selenium compounds excreted into the bile of arsenized rats. Since the bile salts are the major compounds in bile, the study concentrated on these first. Preliminary investigations by Andraos (19) suggested the possible association of selenium with protein; therefore, these compounds were also investigated. Other compounds such as phospholipids, lipids, and cholesterol, were then studied along with smaller metabolites, especially the amino acids to determine if selenium was associated with them.

## MATERIALS AND METHODS

### Apparatus:

The following instruments and apparatus were used in this research: 1) Beckman gamma 4000 counting system, 2) Beckman model 118 BL amino acid analyzer, 3) Beckman model 120 B amino acid analyzer, 4) Waters Model 6000 high performance liquid chromatograph with U6K injector and equipped with a refractory index and ultra-violet detector, 5) Polyacrylamide gel electrophoresis apparatus (vertical) with Gelman variable voltage power source, 6) Refrigeration for Science lyophilization apparatus, 7) Dupont Instruments RC-5 superspeed refrigerated centrifuge, 8) PCR 5000 rotary evaporator, 9) Instrumentation Specialties Co. fractionator, 10) Bausch and Lomb spectronic 88 spectrophotometer, 11) Fisher clinical rotator.

### Materials:

1)  $\text{Na}_2^{75}\text{SeO}_3$  was purchased from ICN Chemical and Radioisotopes Division, 2) Selenomethionine, selenocysteine, cysteine·hydrochloride, glutathione, and other reagents were analytical grade and were used as purchased, 3) Sephadex G-100 and G-200 were obtained from Pharmacia Fine Chemicals, Inc., 4) Silica gel pre-coated thin-layer plates were used for all thin-layer



chromatography and were purchased from Sigma Chemical Co., 5) Cation exchange resin, AG 50W-X8, 20-50 mesh and anion exchange resin, AG 1-X8, 200-400 mesh were purchased from Bio-Rad Laboratories, 6) Sulfoselenocystine (2,7-diamino-4-thia-5-selenaoctanedioic acid), selenocystine and sodium selenite were prepared in this laboratory, 7) Male albino Sprague Dawley rats were purchased from Sasco Inc., Omaha, NE.

#### Special Procedures:

1. Care of the animals: Male Sprague Dawley rats were fed a regular diet of laboratory chow and kept in stainless steel cages at a controlled temperature of 23 C. Food and water were replenished every 48 hours. For experimentation, rats were cannulated at a weight of approximately 350 grams and later terminated by ether inhalation.

2. Cannulation of the bile duct: Cannulation of the bile ducts of rats for the collection of bile was performed after anaesthesia by intraperitoneal injection with sodium pentobarbital (40mg/kg). The abdomen of the rat was shaved and an incision was made along the linea alba. The common bile duct was exposed by pulling on the duodenal loop downward and to the left. A ligature of the common bile duct was tied about 8 mm from its bifurcation. A transverse cut across half of its



diameter was performed above the ligature and a bevelled polyethylene tube, 0.775 mm outside diameter, 0.275 mm internal diameter, and 10-15 cm long, was inserted gently. In order to anchor the catheter in the bile duct, a ligature was tied around the segment of the duct containing the catheter. The operation was then terminated by closing the peritoneum, muscle, and skin. The tube was allowed to protrude between two stitches.

3. Collecton of bile: Bile was collected at half-hour intervals for a period of 3 hours, while the rat was maintained under anesthesia and was immobilized by wrapping in wire mesh. It was collected directly into scintillation tubes. No bile over two weeks old was used as adsorption of selenium to protein was very noticeable. All experiments were run in duplicate unless otherwise indicated.

4. Selenium analysis by gamma radiation: Characterization of the selenium compounds in rat bile was attempted after injecting  $175 \times 10^6$  cpm  $\text{Na}_2^{75}\text{SeO}_3$  per rat. Each rat received 0.5 mg of selenium per kg of rat. Arsenic (1 mg As/kg rat) was injected ten minutes later. Biliary selenite was counted by a gamma scintillation counting system following certain separation procedures. "Control bile" consisted of selenite added in vitro to bile. Selenite (0.1 mg)

labeled with  $60 \times 10^6$  cpm  $\text{Na}_2\text{SeO}_3$  was mixed with 1.0 ml of bile. All separation procedures carried out on metabolized selenite were also performed on control bile.

Characterization of the selenium compounds in rat bile:

Thin-layer Chromatography: Early studies with selenium detected the presence of an unknown selenium metabolite in sheep bile (49) after  $\text{Na}_2^{75}\text{SeO}_3$  was fed in the diets. This compound had a similar  $R_f$  to that of selenotaurine on cellulose thin-layer plates. Since taurine is present in the major bile salt, taurocholate of rat bile, a study to determine whether or not selenium is incorporated with the bile acids was performed.

Aliquots of bile, control bile, and the standards taurocholate, taurochenodeoxycholate, and taurodeoxycholate, were applied to Silica gel thin-layer plates. Three solvent systems were used to develop the thin-layer plates: toluene:acetic acid:water (10:10:1); butanol:acetic acid:water (10:1:1); and chloroform:methanol:acetic acid:water (65:42:15:9). For detection of the bile acids, plates were sprayed with a solution of phosphomolybdic acid (5% w/v) in ethanol/ether (1:1 v/v). The silica gel in two centimeter sections was removed from the plate, counted for activity, and the distribution of radioactivity was compared with the

$R_f$  of the standard bile salts.

High Performance Liquid Chromatography: To confirm the results obtained from thin-layer chromatography, aliquots of bile were injected onto a  $C_{18}$  high performance liquid chromatography column. Fractions were collected manually, counted for activity, and the retention times of  $^{75}\text{Se}$ -containing substances were compared with the retention times of the three major bile salts of rat bile: taurocholate, taurochenodeoxycholate, and taurodeoxycholate.

Conditions were: Column: Alltech  $C_{18}$ ;  
Detector: differential refractometer; Solvent: 80% acetonitrile; flow rate: 2.0 ml/min.

G-200 Gel Filtration: Most selenium in the rat fed a selenium sufficient diet containing ( $^{75}\text{Se}$ ) selenious acid has been found to be associated with protein. In fact, Hawkes, et al. (35) suggested that over 80% of the whole body Se present in the rat is incorporated into protein, especially glutathione peroxidase and that this selenium is present as selenocysteine.

To determine whether or not selenium excreted into bile is associated with protein, bile aliquots were applied to a 1 x 55 cm G-200 (200-400  $\mu\text{m}$ ) Sephadex gel filtration column which was equilibrated in and eluted with 0.05% NaCl. One ml fractions were collected.

Protein in the fractions was identified with Commassie Brilliant Blue binding (54) and the selenium compounds were located by counting radioactivity. The fractions containing activity were compared with those containing protein.

Dialysis: In another attempt to determine the extent of selenium association with protein, protein fractions from gel chromatography were pooled, lyophilized and dialyzed against  $10^{-3}$  M reduced glutathione (GSH). After 24 hours, the GSH solution was replaced by fresh solution for an additional 24 hours. The amount of radioactivity in the GSH solutions was used to determine the percentage of selenium compounds passing through the membrane.

Polyacrylamide Gel Electrophoresis: More specific association of selenium with protein was determined using a 10% acrylamide gel in denaturing and non-denaturing conditions.

Sample Preparation: Protein in bile was denatured by addition of 2-mercaptoethanol and sodium dodecylsulfate (SDS). One ml aliquots of bile were treated (1:1 v/v) with a buffer consisting of 2-mercaptoethanol (1ml), 10% SDS (4ml), glycerol (2ml), and distilled water (3ml). To ensure complete denaturation of protein, samples were heated for 3 minutes at 90 C.

Since both denatured and nondenatured samples were studied, 2-mercaptoethanol and SDS were replaced by water in the treatment buffer of non-denatured samples. Aliquots of 20-50  $\mu$ l were applied to the wells of the stacking gel.

**Gel Development:** The 10% acrylamide gel was developed at 65 mA for 3-4 hours in a buffer containing tris (0.025M pH 8.3), glycine (0.192 M), and SDS (0.1%). The front was marked with bromophenol blue. After development, Comassie Brilliant Blue solution was used to stain the protein. Gels were destained with methanol:acetic acid:water (5:1:4) overnight with gentle shaking on a Fisher clinical rotator.

To determine protein/selenium associations, two centimeter cuts were taken from each gel channel and counted for activity. The areas containing activity were compared with the stained protein areas.

**G-100 Gel Filtration:** Bile contains not only protein and bile salts, but also lipids, phospholipids, cholesterol, and pigments, along with other numerous small metabolites. To separate these major compounds and determine their relationship with selenium, a Sephadex G-100 gel filtration column was employed.

To simplify this matter, Reuben et al. (55) described a physiological eluent similar in electrolyte

composition to rat bile. This solution when used with gel filtration was useful in separating the components of rat bile. These workers showed that the micelles, consisting of lipid, phospholipid, cholesterol, and bile salts could be conserved by utilizing a solution consisting of  $\text{Na}^+$  (125 mM),  $\text{Cl}^-$  (105 mM),  $\text{K}^+$  (5mM), and  $\text{HCO}_3^-$  (25mM). Besides conserving the micelle, its size could be changed by varying the amount of taurocholate added to the eluent. Therefore, to determine whether or not selenium was associated with any of the constituents of the micelle, the elution profile of the micelle was varied and compared with the elution profile of the radioactivity.

Three experiments were performed using a column containing 55 ml of swollen Sephadex G-100 pre-equilibrated at room temperature with three column volumes of the solution subsequently used for elution. In all the experiments, the void volume ( $V_0$ ) was first determined using blue dextran (molecular weight  $2 \times 10^6$ , Pharmacia Fine Chemicals) and the total volume ( $V_t$ ) of the column was measured with bromophenol blue. Bile was applied to the column for descending chromatography and eluate fractions of one ml were collected. These fractions were analyzed for phospholipid, bile pigment, protein, and radioactivity. Since the phospholipid peak is



contained in the mixed-lipid micelles consisting of cholesterol, lipid, and bile salts, these latter substances were not assayed, but assumed to be in the phospholipid fractions.

To vary the size of the bile salt-lipid micelle sufficiently to change its elution profile, different bile salt concentrations were used for column equilibration and elution: 0, 10, and 30 mM taurocholate. The resulting elution patterns of the phospholipids, proteins, and bile pigments were compared with the elution position of the radioactivity.

Extraction of Selenite into Diaminonaphthalene solution:

The possibility of direct secretion of unmetabolized selenite into bile exists; therefore, bile was subjected to a procedure to determine the percentage of selenite in bile (58).

Bile aliquots (20-40  $\mu$ l) were added to a solution of 15 ml of 0.1 M EDTA and 2 ml of 0.1% diaminonaphthalene (DAN) solution. The solution was acidified with 1 ml of perchloric and 0.5 ml of hydrochloric acids. After vortex mixing, the solution was incubated at 60 C for 30 minutes; then cooled for 5 minutes in a running water bath. Cyclohexane (5 ml) was added and the solution was extracted for 5-10 minutes. Selenite present in the solution was determined as the activity

extracting into the cyclohexane layer as a diamino-naphthalene/selenium complex.

Ion Exchange Chromatography:

**Cation Exchange:** An analytical grade cation exchange resin AG 50W-X8, 20-50 mesh ( $H^+$  form in 0.1 M HCl) was used to trap amino acids (45). Fractions at the total volume ( $V_t$ ) off a G-100 Sephadex column were pooled, concentrated under vacuum, and applied to a five ml cation exchange column equilibrated in 0.1 M HCl. The column was washed with 10 ml of 0.1 N HCl and 20 ml of distilled water before the amino acids were eluted with 50 ml of 2 N HCl.

Aliquots of the washings were counted for activity, and the samples were concentrated for amino acid analysis by evaporation under vacuum.

**Anion Exchange:** A similar experiment was performed using an analytical grade anion exchange resin AG 1-X8, 200-400 mesh. Again, fractions at  $V_t$  from G-100 Sephadex were rotary evaporated and applied to a 5 ml column equilibrated in distilled water. Amino acids were removed with 20 ml of distilled water. The column was then washed with 20 ml each of 0.05 M, 1.5 M, and 4.5 M HCl respectively. Aliquots of the washings were counted for activity, and the samples were concentrated for amino acid analysis.



### Amino Acid Analysis on Beckman Model 120 B:

Sample Preparation: Fractions collected at  $V_t$  from G-200 Sephadex gel filtration were pooled, concentrated by evaporation under vacuum, and applied to the cation exchange column to trap amino acids. The fractions containing amino acids were concentrated by rotary evaporation and 0.5 ml fractions were applied to the amino acid analysis column.

Standard amino acid preparation: A solution containing a mixture of selenocystine, cystine, and sulfoselenocystine was prepared by mixing equimolar parts of L-cysteine and D,L-selenocystine at pH 2.2. The mixture was allowed to stand at room temperature for 10 minutes and the pH was adjusted to 7.2 with NaOH. After 1 hour standing with occasional shaking, the pH was adjusted to 2.2. Selenomethionine initially was chromatographed separately, but later was added to the reaction mixture described above.

Chromatographic Methods: The various solutions were chromatographed on a Beckman model 120 B amino acid analyzer. A 50 x 0.9 cm column was packed with Aminex A-4 cation exchange resin (Bio-Rad Laboratories, Richmond California). The buffer flow rate was set at 60 ml/hr, and the temperature of the jacket was set at 50 C throughout each analysis. For elution, Beckman

sodium citrate buffers, pH 3.25 (0.2 N  $\text{Na}^+$ ) and pH 4.25 (0.2 N  $\text{Na}^+$ ) were used. The buffer change was programmed at 91 minutes.

The various selenoamino acid standards were located on the chromatogram by first chromatographing them alone, then later in a mixture. Selenite was located by using the radioactive form by collecting and counting two minute fractions.

Selenium amino acids in bile were chromatographed using the following procedure. The effluent from the column was fed directly to a fraction collector and not allowed to go through the ninhydrin reaction coil. The bile samples were placed on the column, and the fraction collector and elution were begun simultaneously. The fractions collected were assayed for selenium by counting.

The time lag in colorimetric detection and recording of amino acids chromatographed in the usual manner and the appearance of these amino acids in the fractions collected directly from the chromatographic column was determined to be 7 minutes. This time lag was determined in the following way: The effluent during a standard amino acid determination was collected in tubes and ninhydrin solution was added. After heating for 1 hour the absorbance of each tube was read at 570

nm using a spectrophotometer. Ninhydrin positive fractions were compared with the ninhydrin peaks of the chromatogram and the time required for going through the reaction coil was calculated.

Amino Acid Analysis Using Beckman Model 118 B1:

**Sample Preparation:** Samples of freshly collected crude bile and control bile were treated 1:4 (v/v) with sulfosalicylic acid (3.75%) in Beckman lithium citrate buffer (0.2 N Li<sup>+</sup>) adjusted to pH 1.80 with lithium hydroxide. Radioactivities in the supernatant and pellet were counted after centrifugation for ten minutes at 10,000 rpm. The supernatant was filtered through a 0.45  $\mu$ m Micropore filter and 0.5 ml aliquots were applied to the Beckman model 118 B1 amino acid analyzer programmed for physiological amino acids.

Secondly a solution containing 35 standard amino acids was prepared and chromatographed to determine the non-selenium containing amino acids in rat bile.

Finally, the glutathione/selenite compounds in rat bile were determined by mixing equimolar solutions of glutathione and selenite (labeled with Na<sub>2</sub><sup>75</sup>SeO<sub>3</sub>). The solution was allowed to stand for one-half hour with occasional shaking and an appropriate aliquot was diluted (1:4 v/v) with sulfosalicylic acid solution.

**Chromatographic Methods:** The various solutions were chromatographed on a Beckman model 118 BL amino acid analyzer programmed for physiological amino acids. A 0.9 x 18.5 cm column, packed with sulfonated polystyrene cation exchange resin was used. The buffer flow rate was set at 70 ml/hr and the temperature of the jacket varied linearly from 40 C to 62 C. For elution, buffers of pH 2.83 (0.2 N  $\text{Li}^+$ ), pH 3.70 (0.2 N  $\text{Li}^+$ ), and pH 3.75 (1.0 N  $\text{Li}^+$  in 7% 2-propanol) were used. The buffer changes were programmed at 83.7 and 183.8 minutes.

Each sample was applied to the amino acid analyzer and fractions were collected at 1.5 minute intervals after color development by the reaction with ninhydrin. Then, the retention times of the ninhydrin peaks were compared with the retention times of the radioactive fractions. Selenite was located on the chromatogram by collecting fractions and counting the radioactivity in the fractions.

## Results and Discussion

### Characterization of the selenium compounds in bile:

As reported in the literature review, the administration of arsenic after selenium results in the excretion of over 30% of an injected dose in the bile (18,19). This represents a biological system with adequate selenium to permit potential isolation and identification of metabolites. Using bile from rats injected with  $^{75}\text{Se}$ -selenite, and arsenite, several types of characterization studies were performed.

The first of these studies involved examining the possibility of association of selenium with the bile salts. These compounds were studied for two reasons:

1) The bile salts make up over 65% (by dry weight) of all compounds in bile (44). Other compounds in significant quantities include protein (5%), phospholipids (20%), cholesterol (3%), and bilirubin (10%). 2) The association of selenium with, or the substitution for, the sulfur moiety of the bile salts was suspected.

This study was simplified from original designs after it was found that three bile salts make up the majority of all bile salts in rat bile (56).

Taurocholate, the major bile salt, comprises 90% of all bile salts present. Taurocholate, taurochenodeoxycholate

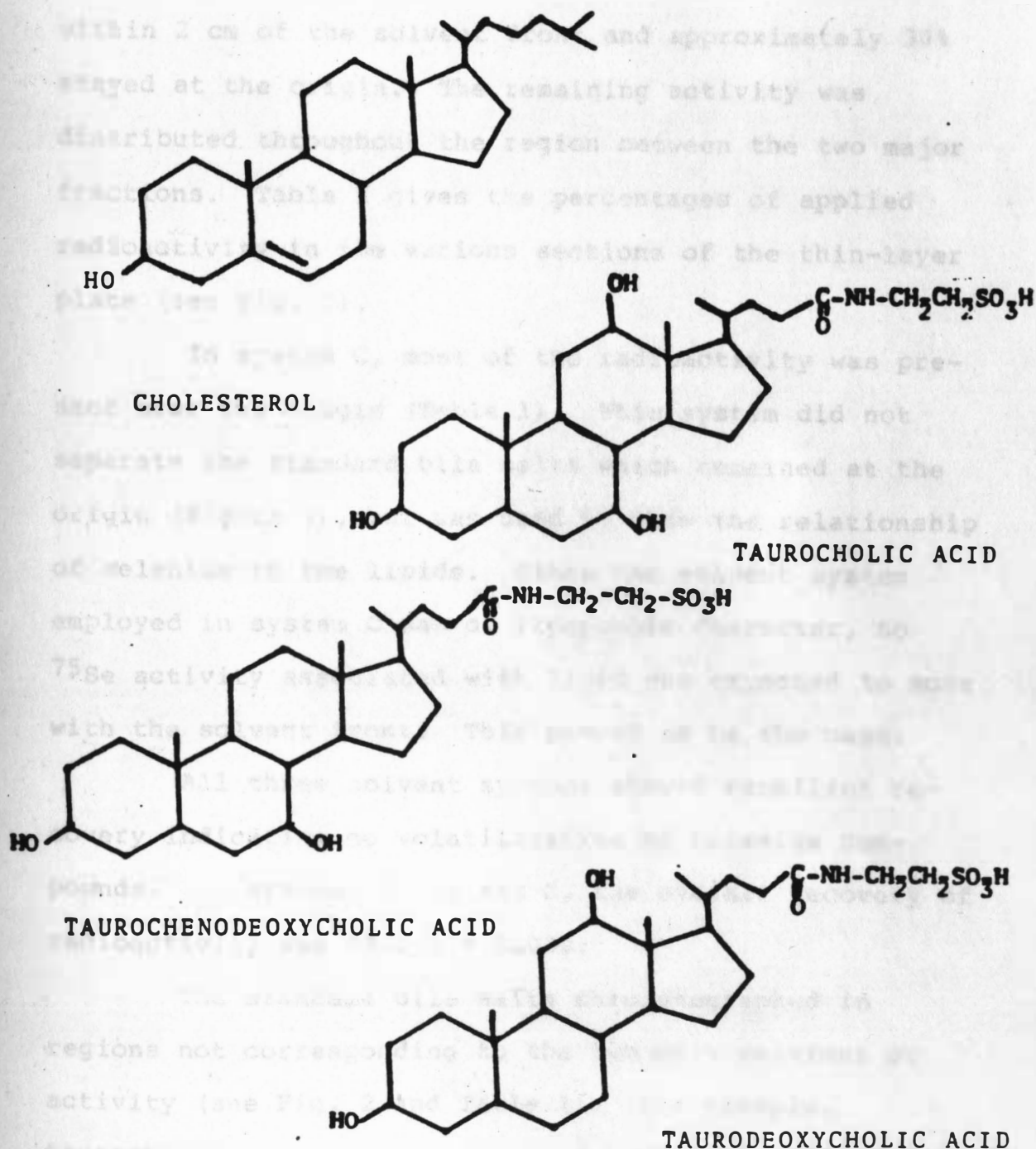
taurodeoxycholate, represent over 97% of all bile salts present. The structures of these compounds can be found in Fig 1.

Because selenium-sulfur interactions are known (57), selenium was suspected to be associated with the taurine conjugate; therefore, experimentation using thin-layer chromatography was done with reference to the three bile salt standards, all of which were conjugates of taurine.

Thin-layer chromatography (TLC): Bile, from  $^{75}\text{Se}$  injected rats, control bile containing added  $^{75}\text{Se}$ -selenite, and the standard bile salts were chromatographed on Silica gel plates. Three solvent systems were used to develop the plates: A) toluene:acetic acid:water (10:10:1); B) chloroform:methanol:acetic acid:water (65:42:15:9); C) butanol:acetic acid:water (10:1:1).

After development and drying, two centimeter sections were scraped from the plates, and the radioactivity was counted. The positions of the radioactivity were compared with the positions of the standard bile salts which were detected by phosphomolybdic acid spray.

Radioactivity was separated into two major fractions on the thin-layer plates. In two systems, systems A & B, 44-53% of the applied radioactivity moved



**Figure 1.** The three major bile acids of rat bile, taurocholic acid, taurochenodeoxycholic acid, and taurodeoxycholic acid, along with the parent compound cholesterol.



within 2 cm of the solvent front and approximately 30% stayed at the origin. The remaining activity was distributed throughout the region between the two major fractions. Table 1 gives the percentages of applied radioactivity in the various sections of the thin-layer plate (see Fig. 2).

In system C, most of the radioactivity was present near the origin (Table 1). This system did not separate the standard bile salts which remained at the origin (Figure 2), but was used to show the relationship of selenium to the lipids. Since the solvent system employed in system C was of lipophobic character, no  $^{75}\text{Se}$  activity associated with lipid was expected to move with the solvent front. This proved to be the case.

All three solvent systems showed excellent recovery indicating no volatilization of selenium compounds. In systems A, B, and C, the overall recovery of radioactivity was  $98.27\% + 3.08\%$ .

The standard bile salts chromatographed in regions not corresponding to the two main portions of activity (see Fig. 2 and Table 1). For example, taurochenodeoxycholate and taurodeoxycholate bile salts moved 6-8 cm from the origin in the toluene system, while taurocholate chromatographed in the region of 4-6 cm (Figure 3). These two sections of the silica gel



Table 1. Thin-layer chromatography of rat bile (both in vivo and in vitro) showing the percentage of radioactivity located in the scrapings from TLC plates.

Solvents <sup>a</sup> :	A		B		C	
	% of Activity		% of Activity		% of Activity	
	<u>In Vivo</u>	<u>In Vitro</u>	<u>In Vivo</u>	<u>In Vitro</u>	<u>In Vivo</u>	<u>In Vitro</u>
<u>Distance from origin (cm)</u>						
0-2	27.18	94.37	28.69	92.40	28.04	75.50
2-4	2.29	0.49	5.55	0.82	14.42	17.42
4-6	1.35	0.26	3.43	0.47	15.44	10.10
6-8	2.01	0.17	1.84	0.37	15.68	0.40
8-10	6.15	0.15	2.13	0.26	15.39	0.15
10-12	6.06	0.25	2.26	0.15	8.17	0.10
12-14	52.94	0.31	9.44	0.21	0.65	0.12
14-16	---	---	44.35	0.40	1.20	0.09
Recovery:	97.98	96.00	97.69	95.08	98.99	103.88
Overall recovery:	98.27 + 3.08					

<sup>a</sup>Three solvent systems were used: A) toluene:acetic acid:water (10:10:1); B) chloroform:methanol:acetic acid:water (65:42:15:9); and C) butanol:acetic acid:water (10:1:1).

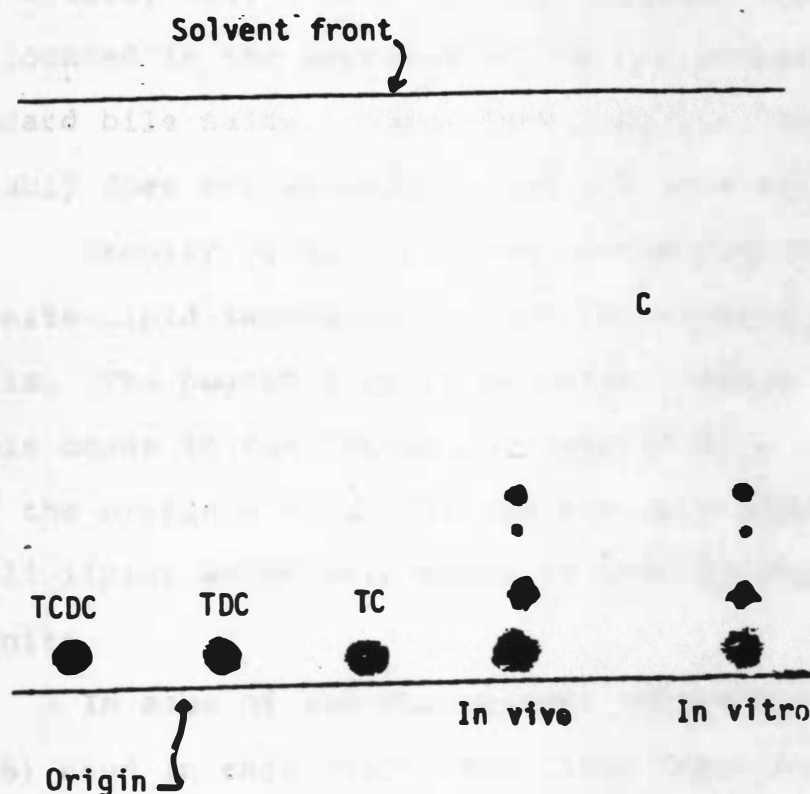


Figure 2. Thin-layer chromatogram of the bile salts of rat bile in a less lypophylic solvent. Most of the radioactivity appeared at the origin indicating a possible association of selenium with lipid material. The three bile salts, taurochenodeoxycholic (TCDC), taurodeoxycholic (TDC), and taurocholic (TC) salts stayed at the origin. The solvent used was butanol:acetic acid:water (10:1:1).

included 3.36% of the activity applied. In the chloroform system, less than 5% of the applied radioactivity was located in the sections of the gel containing the standard bile salts. These data indicate that selenium probably does not associate with the bile salts.

Gennity et al. (53) has recently discussed a selenite-lipid interaction in marine animals and plants. The possibility of selenite binding to the double bonds in the lipids was suggested by the finding that the olefinic structure was the only feature common to all lipids which were found to contain selenium from selenite.

In some of the TLC systems (Table 1, solvents A and B) used in this study, the lipid fraction would be expected to move toward the front. Therefore, it is possible that the  $^{75}\text{Se}$  activity which moved toward the front is associated with the lipid. Perhaps this association is with the olefinic structures as suggested by Gennity et al. (53). Another 30% of the applied  $^{75}\text{Se}$ -radioactivity did not move far from the origin in all 3 of the solvent systems (Table 2.). This activity may represent unreacted selenite since this is where the major portion of the activity in the in vitro bile moved. It is also possible that some of the activity

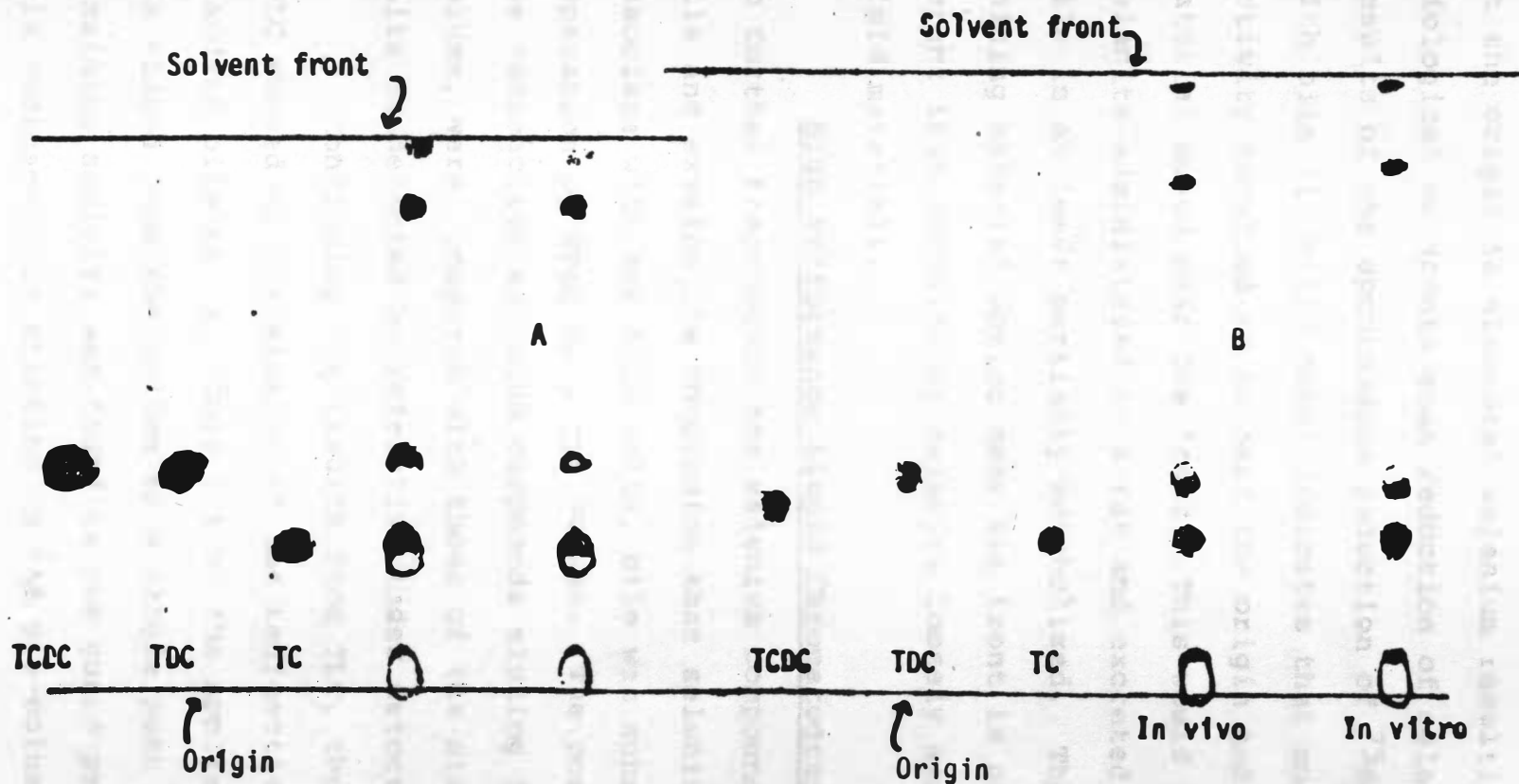


Figure 3. Thin-layer chromatogram of the bile salts of rat bile. Three standard bile salt, taurochenodeoxycholic (TCDC), taurodeoxycholic (TDC), and taurocholic (TC) salts along with in vivo and in vitro bile were chromatographed on Silica gel thin-layer plates. In vivo bile consisted of bile collected after the injection of radioactive selenite (0.5 mg Se/kg rat) followed by arsenite (1 mg As/kg rat). In vitro, or control bile consisted of radioactive selenite (0.1 mg Se) added to 1 ml of bile after collection. Two types of solvents were used to develop the plates: A) toluene:acetic acid:water (10:10:1); B) chloroform:methanol:acetic acid:water (65:42:15:9).

at the origin is elemental selenium resulting from the biological or spontaneous reduction of selenite. The results of the spontaneous reduction of  $^{75}\text{Se}$ -selenite with bile (in vitro data) indicates that most of the activity remained at or near the origin and very little material moved near the front. This would indicate that selenite administered to a rat and excreted in the bile is at least partially metabolized. The  $^{75}\text{Se}$  containing material moving near the front is certainly different than selenite or selenite loosely associated with lipid materials.

#### High Performance Liquid Chromatography (HPLC):

To further fractionate the selenium compounds in rat bile and examine the conclusion that selenium does not associate with the bile salts, bile was submitted to separation by HPLC on a C18 column. The positions of the radioactive selenium compounds eluting from the column, were compared with those of the standard bile salts as detected by refractive index detection.

Confirming the results from TLC, the data from HPLC showed no correlation of the radioactivity with the standard bile salts. Only 60% of the applied activity was eluted from the column as a single peak while the remaining activity was found in the guard pre-column. This radioactivity sticking to the pre-column suggests

the selenium was associated with protein or that selenite was reduced to elemental selenium. Neither form would be expected to pass through the column. All of the radioactivity passing through the column occurred as a single peak that corresponded with the injection peak. This is also where selenite eluted. The bile salts eluted much later. Other lipid material would also be expected to be eluted more slowly on HPLC and so these data contradicted the suggestion, made previously, that selenium in bile is associated with lipids and their olefinic structures.

Since a major portion of the activity was lost to the pre-column, selenium/protein association was suspected. To determine the extent of this association, a G-200 Sephadex gel filtration column was used to fractionate the proteins.

G-200 Sephadex gel filtration: Again, bile and control bile were studied. Aliquots of each were applied to a 1 x 55 cm column which was eluted with 0.05% NaCl, and one ml fractions were collected. Each tube was assayed for protein (54) and counted for gamma emission. Those tubes containing the bulk of the protein were compared with the tubes containing the major portion of radioactivity.

Only three chromatographic forms of selenium were found in the gel filtration studies. The first form was measured as the  $^{75}\text{Se}$  remaining in the top few centimeters of the gel after completion of the run. This accounted for 30% of the applied radioactivity and probably represented a labile form which decomposed since elemental selenium was observed at this position after several chromatographic runs.

A second fraction was found associated with protein at the void volume (Fig. 4). This fraction would contain the very large proteins (Fig. 4) and the associated  $^{75}\text{Se}$ -activity represented less than 10% of the radioactivity applied to the column. When samples of the same bile were chromatographed at a later date, a greater portion of the activity was found in the protein fractions; in fact, up to 50% of the applied radioactivity could be found in these fractions after one week. Since the  $^{75}\text{Se}$  activity in this fraction increased with time, this probably represented a simple reaction of the sulfhydryl groups of protein with selenite. Selenium has been found notorious for this type of interaction (20,21).

Finally, a third portion was found in the fractions representing the total volume (Fig. 4). This is the position where the selenite standard was also found.



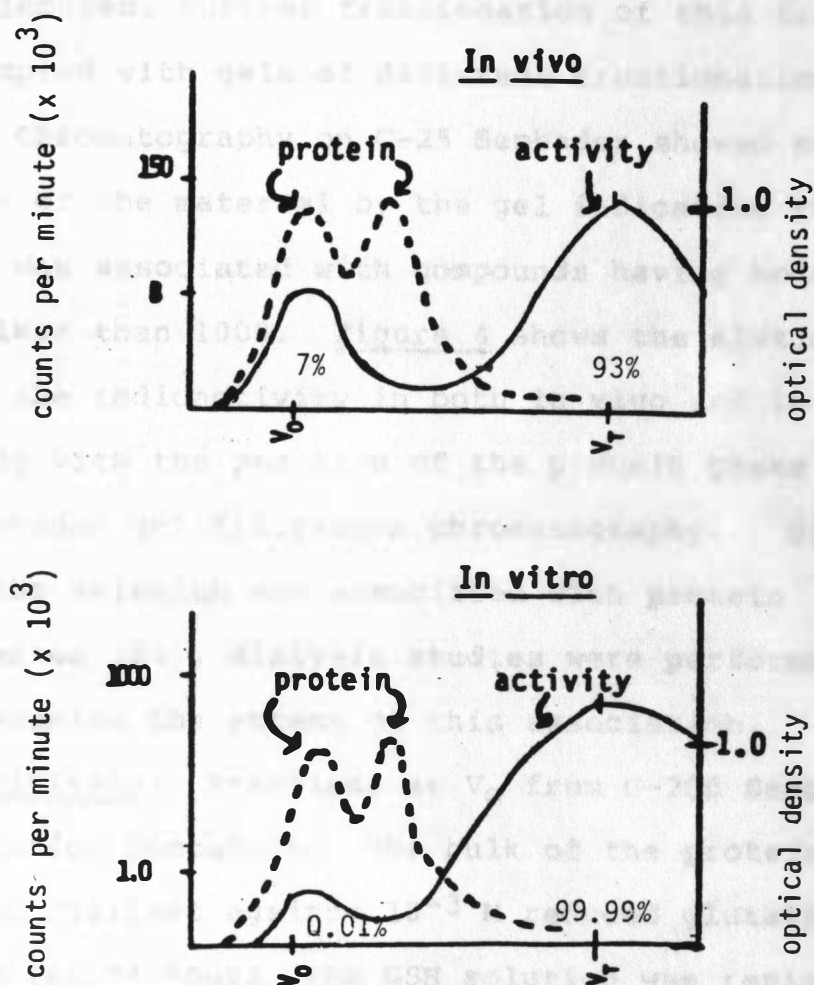


Figure 4. G-200 Sephadex gel filtration chromatography of rat bile in 0.05% NaCl aqueous solution. Both in vivo (top) and in vitro (bottom) bile samples were studied. The relationship of the two protein peaks to the radioactive peaks is shown. Selenium present at  $V_0$  may be due to protein association. The percentages represent the percent of applied radioactivity.



Since the substances in this fraction would be relatively small molecules, further fractionation of this fraction was attempted with gels of different fractionation ranges. Chromatography on G-25 Sephadex showed no fractionation of the material by the gel indicating the selenium was associated with compounds having molecular weights less than 1000. Figure 4 shows the elution profiles of the radioactivity in both in vivo and in vitro bile along with the position of the protein peaks from G-200 Sephadex gel filtration chromatography. Since some of the selenium was associated with protein (approximated 10%), dialysis studies were performed to further examine the extent of this association.

Dialysis: Fractions at  $V_0$  from G-200 Sephadex gel filtration containing the bulk of the protein were pooled and dialyzed against  $10^{-3}$  M reduced glutathione (GSH). After 24 hours, the GSH solution was replaced by a fresh solution and dialysis continued for another 24 hours. In the first dialysate, 45% of the original activity was removed and another 25% was removed following fresh buffer. Since most of the selenium could be removed by dialysis, protein/selenium associations seemed very non-specific, however, further studies on protein-selenium association were performed. These data support the earlier observation of Levander

and Baumann (18).

Polyacrylamide gel electrophoresis: Both denaturing and non-denaturing conditions were used when studying protein fractionation in the polyacrylamide gels. When aliquots of bile protein were developed without protein denaturation, 14 distinct polypeptide bands (Figure 5) were observed, but little radioactivity was associated specifically with these bands. Of the radioactivity applied, 65% moved into the buffer cell at the origin. This direction of movement would make this fraction of the radioactivity appear to be relatively small, neutral or positively charged molecules. Another 30% of the radioactivity was recovered in the gel, but it was all essentially located at the origin.

In denaturing conditions (Figure 5.), 24 polypeptide bands could be observed, but again no specific association of the radioactivity with the bands was observed. Of the radioactivity applied, over 90% was associated with the gel at the bromophenol front. The rest of the radioactivity was distributed evenly throughout the gel and very little was in the buffers.

When the results of the gel filtration, dialysis, and electrophoresis experiments are compared, it appears that all support the conclusion that selenium is not incorporated into the protein. Consequently, the study

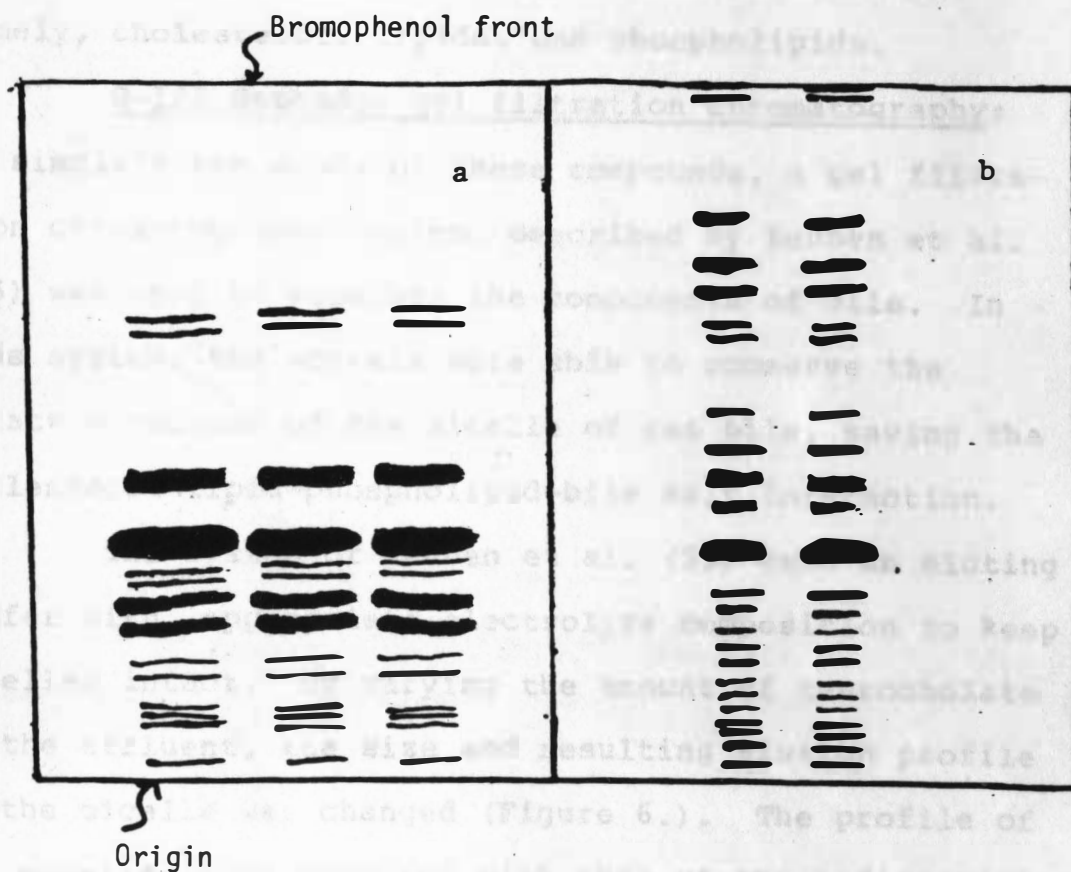


Figure 5. Polyacrylamide gel electrophoresis of the proteins in rat bile. a) The distribution of the 14 protein bands in non-denaturing conditions. Selenium radioactivity was present in the buffer located at the origin (65%) and at the origin (30%). b) The distribution of the 24 protein bands in denaturing conditions. Selenium radioactivity was present for the most part at the solvent front (90%). Most of the other radioactivity was distributed evenly throughout the gel.

was then directed toward other components in bile; namely, cholesterol, lipids, and phospholipids.

G-100 Sephadex gel filtration chromatography:

To simplify the study of these compounds, a gel filtration chromatography system, described by Reuben et al. (55) was used to separate the components of bile. In this system, the workers were able to conserve the intact structure of the micelle of rat bile, saving the cholesterol-lipid-phospholipid-bile salt interaction.

The system of Reuben et al. (55) used an eluting buffer with appropriate electrolyte composition to keep micelles intact. By varying the amount of taurocholate in the effluent, the size and resulting elution profile of the micelle was changed (Figure 6.). The profile of the micelles were compared with that of the radioactivity to determine whether or not the selenium compounds of rat bile were associated with any of the components of the micelle.

In 30mM taurocholate, the position of the mixed micelle peak was at  $1/2 V_T$  (see top part of Fig. 6). When the taurocholate concentration was reduced to 10 mM (middle of Fig. 6), the elution volume of the micelles decreased to just slightly greater than  $V_0$  and when taurocholate was omitted, (bottom of Fig. 6), the  $V_e$  of the micelles was identical to  $V_0$ .

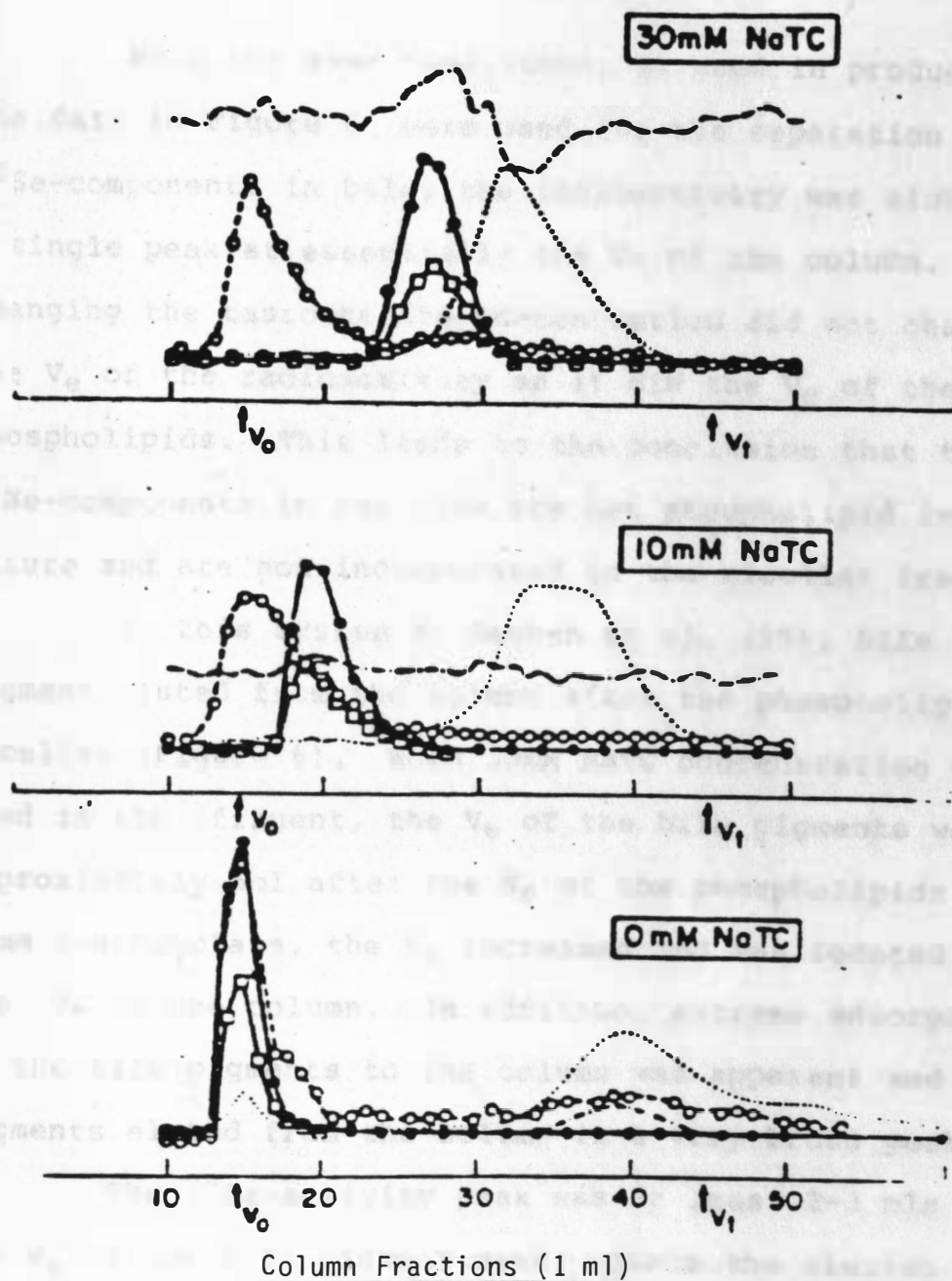


Figure 6. G-100 Sephadex gel filtration chromatography of rat bile (55). Samples of bile were applied to columns that had been pre-equilibrated and were subsequently eluted with bile salt-electrolyte solutions containing 30 mM (top), 10 mM (middle), and 0 mM (bottom) sodium taurocholate (NaTC). Phospholipid (—●—), cholesterol (—○—), bile salt (---○---), protein (···○···), and bile pigment (····○····) peaks are shown. The position of the phospholipid peak was used as a marker of the elution volume of the mixed lipid micelles in bile.

When the same conditions, as used in producing the data in Figure 6, were used for the separation of  $^{75}\text{Se}$ -components in bile, the radioactivity was eluted as a single peak at essentially the  $V_t$  of the column. Changing the taurocholate concentration did not change the  $V_e$  of the radioactivity as it did the  $V_e$  of the phospholipids. This leads to the conclusion that the  $^{75}\text{Se}$ -components in rat bile are not phospholipid in nature and are not incorporated in the micellar fraction.

In this system of Reuben et al. (55), bile pigment eluted from the column after the phospholipid micelles (Figure 6). When 30mM NaTC concentration was used in the effluent, the  $V_e$  of the bile pigments was approximately 5ml after the  $V_e$  of the phospholipids. In 0 mM taurocholate, the  $V_e$  increased and was located near the  $V_t$  of the column. In addition, extreme adsorption of the bile pigments to the column was apparent and these pigments eluted from the column in a very broad peak.

The  $^{75}\text{Se}$ -activity peak was at least 2-3 mls after the  $V_e$  of the bile pigment peak. Since the elution volumes of the radioactivity and the bile pigments did not correlate, these data suggest that selenium in bile is not covalently associated with the bile pigments. However, the  $V_e$  of the  $^{75}\text{Se}$ -activity peak appeared to decrease in 30mM taurocholate in conjunction with

the  $V_e$  of the bile pigment peak. These data suggest a non-specific association of selenium (as selenite) with the bile pigments as described by Gennity et al. (53), but do not explain the lack of association of  $^{75}\text{Se}$ -activity with the lipids. Possibly the selenium/pigment association is greater than the selenium/lipid association.

Since the major portion of the selenium compounds in bile appeared to be small metabolites, the remainder of the study was directed toward identifying whether selenite, selenate, or selenoamino acids were present in bile.

Ion exchange chromatography: To further examine the nature of the small selenium metabolites, two ion exchange experiments were performed. In the first experiment a cation exchange procedure described by Hawkes et al. (45), was used to trap amino acids. Fractions at  $V_t$  from G-100 Sephadex gel filtration (small molecules) were pooled, concentrated by evaporation under vacuum, and applied to a column of AG-50 W-X8, 20-50 mesh ( $\text{H}^+$  form in  $\text{HCl}$ ). The column was washed with 10 ml of 0.1 N  $\text{HCl}$  and 20 ml of distilled water to remove anionic compounds including selenite and selenate. Amino acids were then eluted with 2 N  $\text{HCl}$ . Finally the column was washed with 6 N  $\text{HCl}$  to remove any



other material (such as trimethylselenonium ion (10)). The percentage of radioactivity in each elution solution was then determined. Table 2 gives the percentage of radioactivity in each of the solutions and indicates which fractions correspond to selenite and selenoamino acids.

Total recovery from the cation exchange column was approximately 73%. Of the radioactivity applied, 25% was removed with the first two washings (0.1 N HCl and H<sub>2</sub>O). This probably represented the selenite and selenate present in addition to any other anions or neutral materials. Another 43% was removed with 2 N HCl. This indicates the maximum amount present as selenoamino acids in the washings of the cation exchange column. Since the liver is generally assumed to be a reductive organ relative to selenium metabolism, selenate, an oxidative product of selenite, was probably not present.

A further attempt to fractionate the selenium compounds in the bile of arsenized rats was made using an anion exchange column, AG 1X-8, 200-400 mesh. Again fractions at  $V_t$  from G-100 Sephadex gel filtration were rotary evaporated and applied to a 5 ml column. The resin was equilibrated with distilled water, and washed with 20 ml of each of the following solvents: distilled water, 0.05 N HCl, 1.5 N HCl, and 4.5 N HCl respectively.

Table 2. Ion exchange chromatography of the total volume fractions from G-100 Sephadex gel filtration of rat bile.

AG 50W-X8 Cation Exchange Chromatography

<u>Effluents:</u>	<u>Radioactivity:</u>	<u>% in Fraction:</u>
10 ml of 0.1 N HCl <sup>ab</sup>	45,983 cpm	20.91
20 ml of H <sub>2</sub> O	9,995 cpm	4.55
50 ml of 2.0 N HCl <sup>c</sup>	93,674 cpm	42.61
20 ml of 6.0 N HCl	9,657 cpm	<u>4.39</u>
Total:		72.46

AG 1X-8 Anion Exchange Chromatography

<u>Effluents:</u>	<u>Radioactivity:</u>	<u>% in Fraction:</u>
50 ml of H <sub>2</sub> O <sup>c</sup>	155,015 cpm	43.08
50 ml of 0.05 N HCl <sup>a</sup>	149,458 cpm	41.54
50 ml of 1.50 N HCl <sup>b</sup>	9,786 cpm	2.72
50 ml of 4.50 N HCl	7,137 cpm	<u>1.98</u>
Total:		89.32

<sup>a</sup>Represents the fractions containing selenite. <sup>b</sup>Represents the fractions containing selenate. <sup>c</sup>Represents the fractions containing the amino acids.

Two major peaks of radioactivity were eluted from the column. The first came off with the water and corresponded to where selenomethionine would elute. Approximately 43.1% of the applied activity was in this fraction. The second major peak of activity occurred in the 0.05 N HCl fraction. This corresponds to where selenite would elute and accounted for 41.5% of the total. The 1.50 N HCl fraction, in which selenate would elute, accounted for no more than 2.7%.

Since both ion exchange experiments suggested the existence of selenite and selenoamino acids in rat bile, both of these compounds were studied further.

Extraction of selenite into DAN solution: To determine the percentage of unmetabolized selenite in bile, bile aliquots were added to a solution of EDTA and diaminonaphthalene (DAN) solution. Since DAN is known to react with only SeIV (58), the amount of selenite in bile was determined by extraction of the DAN/selenite complex into cyclohexane. The amount of radioactivity in the cyclohexane layer was then counted.

Although the ion exchange experiments had indicated the presence of a large percentage of selenite in bile the extraction experiment suggested differently (Table 3). Only 5% of the radioactivity extracted into the upper cyclohexane layer as the DAN/selenite

Table 3. Determination of the percentage of selenite<sup>a</sup> in bile and in the total volume fractions (V<sub>t</sub>) from G-100 Sephadex gel filtration (both in vivo and in vitro).

	<u>Bile</u>		<u>V<sub>t</sub> fractions from G-100</u>	
	<u>In Vivo</u>	<u>In Vitro</u>	<u>In Vivo</u>	<u>In Vitro</u>
% of Selenite <sup>b</sup> :	5.19 ± 4.02	71.94 ± 2.80	29.39 ± 1.54	68.77 ± 2.83
% of Other Selenium Compounds <sup>b</sup> :	37.39 ± 2.72	31.75 ± 3.25	62.50 ± 3.56	27.86 ± 2.02
% Recovery:	42.58 ± 3.11	103.69 ± 4.20	91.89 ± 2.65	96.63 ± 1.68

<sup>a</sup>The percentage of selenite in bile was determined by extracting selenite as a DAN/selenite complex into cyclohexane. <sup>b</sup>Radioactivity extracting into cyclohexane indicated selenite. <sup>c</sup>Radioactivity remaining in the aqueous layer was assumed to be other selenium compounds.

complex. Another 35% was left in the lower aqueous layer. This represented only 40% of the radioactivity initially present. The lost radioactivity was located between the cyclohexane/aqueous layers present in an emulsion.

Control bile samples were also subjected to this procedure to see whether or not selenite would be trapped in an emulsion. Selenite (0.1 mg) was added to 1 ml of bile. The bile sample was then subjected to the extraction procedure. Selenite added to bile in vitro did not become trapped in an emulsion, but some selenite reacted with an aqueous soluble compound since 30% of the radioactivity was found in this fraction. Only 70% of the selenite was extracted into cyclohexane as a DAN-selenite complex or piasselenol.

The nature of the selenium trapped in the emulsion layer is unclear. It may be that selenite is associated with some organic moiety, in the process of excretion in the bile, which makes it inaccessible to reaction with DAN. If so, this would be an enzymatically catalyzed reaction, since it did not occur when selenite was just added to bile (in vitro bile). The nature of the Se material in the emulsion layer remains to be determined.

Finally, fractions at  $V_t$  from G-100 Sephadex gel

filtration, which contained small molecular weight metabolites, were also studied for the presence of selenite (Table 3). Again, most of the activity did not extract into cyclohexane as the DAN/selenite complex. Only 30% of the activity extracted, while another 70% stayed in the lower aqueous layer. The data from this experiment agrees with those obtained from cation exchange experiments, since 30% of the radioactivity appears as selenite.

Besides selenite, selenoamino acids were also suspected to be present in rat bile; therefore, studies to isolate and quantitate these compounds were done.

Amino acid analysis on Beckman model 120B: Preliminary studies were performed on a Beckman model 120B to identify any selenoamino acids in rat bile. Fractions at  $V_t$  from G-100 Sephadex were pooled, evaporated under vacuum and applied to a cation exchange column to trap amino acids (45). Fractions containing the amino acids were applied to the amino acid analyzer and 2 minute fractions were collected. The radioactivity in each tube was counted. The retention times of the activity were compared with the retention times of some known selenoamino acids.

Figure 7 is a schematic of the chromatogram of

# AMINO ACID ANALYSIS

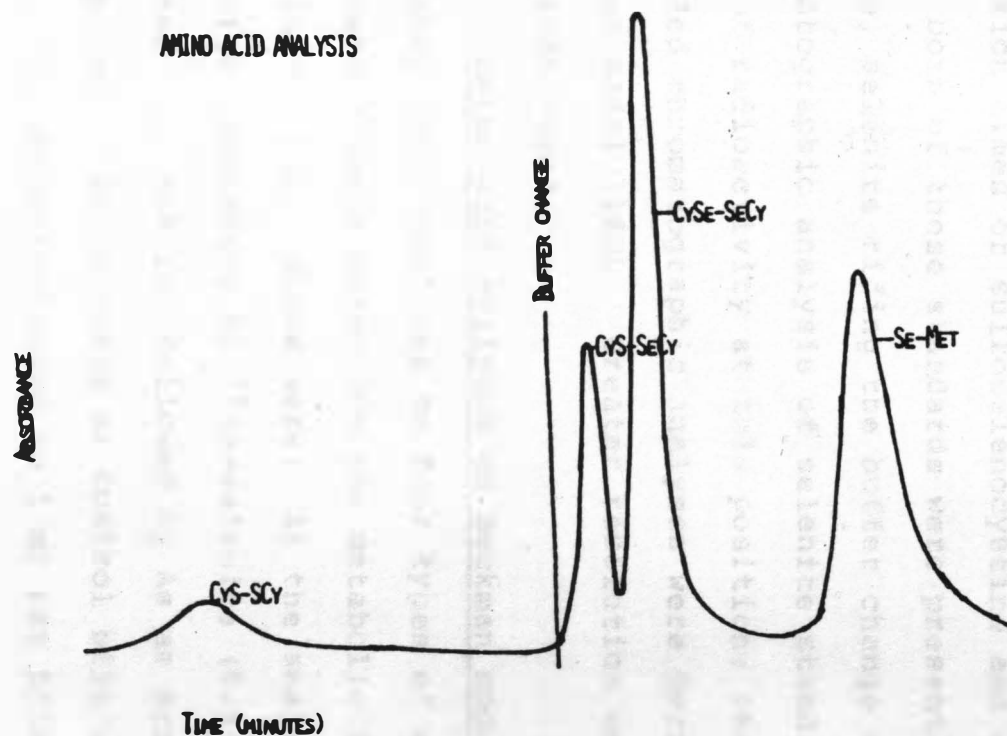


Figure 7. Representation of the chromatogram of some common sulfur and selenoamino acids; cystine, selenocystine, sulfoselenocystine, and selenomethionine. Two radioactive peaks occurred at the position of sulfoselenocystine and selenocystine, immediately after the buffer change.



four amino acid standards cystine, selenocystine, sulfo-selenocystine, and selenomethionine. Two radioactive peaks were obtained in the effluent from the amino acid analyzer. Their retention times correlated with the retention times of sulfoselenocystine and selenocystine. Since both of these standards were present near a buffer change, selenite riding the buffer change was suspected. Chromatographic analysis of selenite standard showed no peak of radioactivity at this position; therefore, more detailed chromatographic analyses were performed on a Beckman model 118BL. Greater resolution was possible on this instrument.

Amino acid analyses on Beckman model 118BL:

Chromatographic analyses on four types of samples were performed to help determine the metabolic fate of selenite in the rat. These were: 1) the analysis of in vivo bile consisting of  $^{75}\text{Se}$ -selenite (0.5 mg/kg) injected into the rat followed by As as arsenite (1.0 mg/kg); 2) in vitro or control bile consisting of 1.0 mg  $^{75}\text{Se}$ -selenite added to 1 ml rat bile after collection; 3) a glutathione/selenite mixture consisting of equimolar solutions of reduced glutathione (GSH) and  $^{75}\text{Se}$ -selenite; 4) a standard amino acid solution consisting of 38 amino acids listed in Table 4.

In the first two analyses, in vivo and in vitro

bile, the protein was precipitated and centrifuged as described in the methods section. Approximately 90% of the radioactivity was present in the supernatant, while the remainder of the radioactivity was associated with the protein pellet. The supernatant liquids of these samples, and the glutathione/selenite and standard amino acid solutions were each applied to a column of a Beckman model 118BL amino acid analyzer. Aliquots of 0.5ml were used. After ninhydrin detection of the amino acids and polypeptides, fractions were collected at 1.5 minute intervals. These fractions were then counted for  $^{75}\text{Se}$ -activity. Therefore, both a chromatogram of the ninhydrin positive peaks and an elution profile of the radioactivity were obtained. The chromatograms (Figure 8) and elution profiles of the radioactivity (Figure 9) of each sample were compared to help determine the extent of the metabolism of selenite in arsenic-treated rats.

Comparison of the chromatograms of *in vivo* and *in vitro* bile (Fig. 8, b and c) showed that these two bile samples were basically identical in composition of the ninhydrin positive material. This indicates that no different selenoamino acids or selenopolypeptides were formed or excreted in bile when selenite was injected into rats, as compared to when selenite was simply added

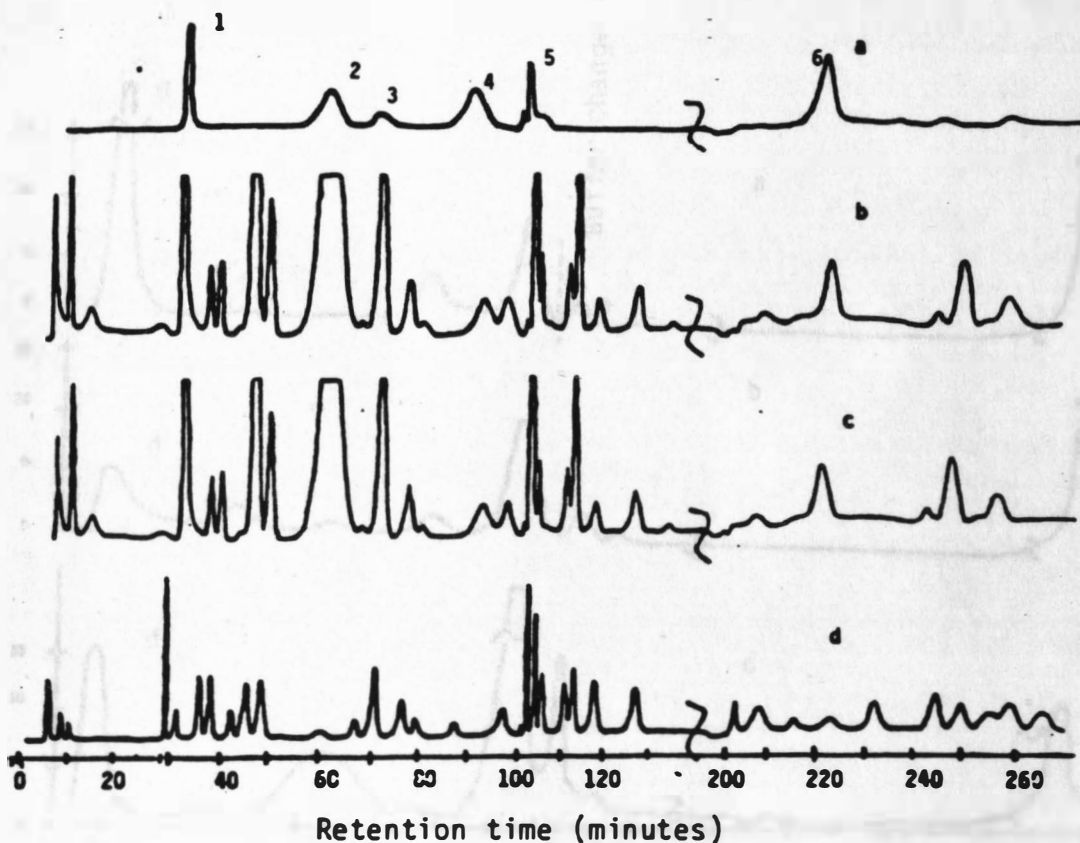


Figure 8. A comparison of four amino acid analyses:  
 a) A mixture of equimolar solutions of GSH and selenite consisting of 1) GSH; 2) GSSG; 3) GSSeH; 4) GSSeSG; 5) a degradation product of glutathione; and 6) ammonia.  
 b) Rat bile with selenite added in vivo.  
 c) Rat bile with selenite added in vitro.  
 d) Several standard amino acids listed in Table 4.

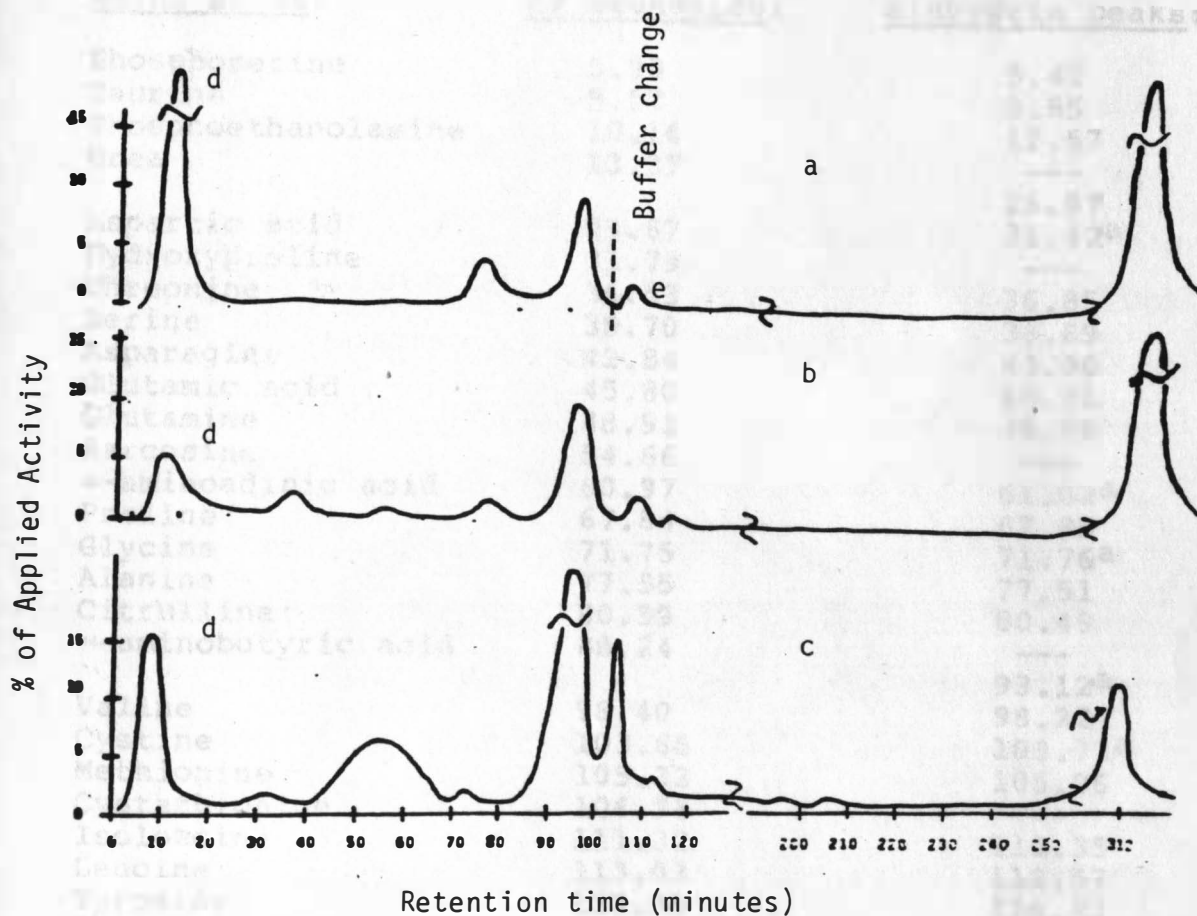


Figure 9. The percentage of radioactivity in the fractions collected from amino acid analysis and the retention times of these radioactive peaks are shown. A comparison of three analyses is illustrated:

- a) A mixture of equimolar solutions of glutathione and selenite;
- b) Rat bile with selenite added in vivo; c) Rat bile with selenite added in vitro; d) Selenite eluted at a retention time of approximately 6 minutes, correlating with the first peak eluting from the column; e) Sulfoselenocystine eluted immediately after the buffer change.

Table 4. The retention times of the standard amino acids and a comparison to some ninhydrin peaks of bile.

<u>Amino acids:</u>	<u>Retention times of standards:</u>	<u>Retention times of ninhydrin peaks:</u>
Phosphoserine	5.80	5.42
Taurine	8.57	8.55
Phosphoethanolamine	10.16	12.57
Urea	13.37	---
		26.97
Aspartic acid	29.67	31.42 <sup>a</sup>
Hydroxyproline	31.79	---
Threonine	36.53	36.85
Serine	38.70	38.89
Asparagine	42.84	43.00
Glutamic acid	45.80	45.96
Glutamine	48.91	48.98
Sarcosine	54.66	---
$\alpha$ -aminoadipic acid	60.97	61.02 <sup>a</sup>
Proline	67.84	67.85
Glycine	71.75	71.76 <sup>a</sup>
Alanine	77.55	77.51
Citrulline	80.59	80.49
$\alpha$ -aminobutyric acid	88.24	---
		93.12 <sup>a</sup>
Valine	98.40	98.22
Cystine	103.66	103.73 <sup>a</sup>
Methionine	105.32	105.06
Cystathionine	106.76	---
Isoleucine	111.32	110.35
Leucine	113.03	112.57
Tyrosine	117.35	116.83
Phenylalanine	125.69	125.01
		132.05
Homocystine	173.55	---
$\gamma$ -aminobutyric acid	202.93	---
Tryptophane	207.57	206.86
Ethanolamine	215.10	---
Ammonia	222.49	220.33 <sup>a</sup>
Hydroxylysines	231.31	---
Ornithine	243.95	242.40
Lysine	249.09	247.52
1-methylhistidine	254.98	---
Histidine	258.87	256.87
3-methylhistidine	265.72	---
Arginine	295.97	293.59

<sup>a</sup>The mixture of GSH and selenite had ninhydrin peaks in these areas.

to bile after collection (in vitro).

In agreement with these chromatographic (ninhydrin detection) data, observation of the  $^{75}\text{Se}$ -activity elution profiles of the two bile samples led to the same conclusion. When fractionation of the radioactivity of in vivo bile was done, 8 radioactive peaks were found (Fig. 9, b). Although the percentages in each fraction were different (Table 5), all of these radioactive peaks could be accounted for by substances formed in in vitro bile (Fig. 9, b and c). Therefore, in vivo and in vitro bile again appeared very similar.

Two of the radioactive peaks of in vivo bile could be accounted for by standards. For example, selenite standard was found to have a retention time identical with the first peak (Table 5, Figure 9, b). This peak eluted at a retention time of 6 minutes. Another radioactive peak was detected at a retention time of 105 minutes (Table 5). Although this is the same retention time as observed for standard sulfoselenocystine (see footnote b, Table 5), it should be noted that a similar peak was observed in the analysis of the reaction mixture of GSH and selenite (peak 5, Fig. 8, a). Since one would not expect sulfoselenocystine to be formed under the latter condition, it raises doubt as to whether the peaks in the bile samples at this position is due to

sulfoselenocystine. A more likely explanation of the activity at 105 minutes is that it represents selenite riding the buffer change. No peak of activity was observed in this region during chromatographic analysis of selenite standard, but streaming of activity throughout the column was present. Therefore, this radioactive peak near the buffer change could be accounted for by the nonspecific association of selenium (as selenite) with some compound (possibly degradation products) eluting at this position.

A selenomethionine standard was shown to have a retention time of 118 minutes under the same conditions. None of the retention times of the radioactive ( $^{75}\text{Se}$ ) peaks in the bile samples corresponded to this value.

Other radioactive peaks from the chromatographic analysis of rat bile (in vivo) could be accounted for by the glutathione/selenite standard solution.

Glutathione, both reduced (GSH) and oxidized (GSSG) forms is known to be present in rat bile (22). Chromatographic analysis of individual standard samples of GSH and GSSG showed that these two compounds had retention times of 30 and 60 minutes respectively (Fig. 8, a, peaks 1, 2). Another degradation product of standard glutathione was shown to be present at 105 minutes (Fig. 8, a, peak 5). These two peaks (1 and 2)



Table 5. The percentages of radioactivity present in fractions taken from amino acid analysis.

<u>Retention times (min):</u>	<u>% Radioactivity:</u>		
	<u>In vivo</u> <sup>c</sup>	<u>In vitro</u> <sup>d</sup>	<u>Glutathione</u> <sup>e</sup>
6 <sup>a</sup>	5.0	15.0	27.4
33	2.2	1.5	---
51	1.2	5.6	---
70.5	1.3	1.1	4.2
91.5	10.0	45.2	9.7
105 <sup>b</sup>	2.2	9.5	1.4
112.5	0.9	3.4	---
192	---	0.13	---
204	---	0.36	---
312 <sup>f</sup>	68.8	10.5	33.2

<sup>a</sup>Position of selenite as shown by standard.

<sup>b</sup>Position of sulfoselenocystine as shown by standard.

<sup>c</sup>Selenite was injected into the rat and bile was collected for amino acid analysis.

<sup>d</sup>Selenite was added to bile after collection.

<sup>e</sup>Selenite was added to glutathione (1:1 mole/mole).

<sup>f</sup>The LiOH fraction from amino acid analysis.

correlated with the retention times of two major peaks of rat bile (Fig. 8, a and b). This suggests high concentrations of glutathione in bile which has been reported previously (22).

Chromatographic analysis of the glutathione/selenite mixture showed 6 ninhydrin positive peaks (Fig. 8, a). Peaks 1, 2, 5 and 6 could be accounted for by chromatographing standards. These were GSH, GSSG, a degradation product of glutathione emerging at the buffer change, and ammonia respectively. The other two peaks (peaks 3 and 4) could not be accounted for directly by standards, but since they were products of the reaction of selenium with glutathione, certain inferences can be made from the literature. From the work of Ganther (20, 21), it would appear likely that these substances are GSSeSG and GSSeH.

After chromatographic analysis of the mixture, the fractions containing peaks 3 and 4 (determined by ninhydrin detection) proved to contain  $^{75}\text{Se}$ -activity. The retention times of these glutathione/selenite peaks correlated with two radioactive peaks of rat bile in vivo (Fig. 8, a and b).

The major peak (peak 4) contained 10% of the radioactivity of the GSH/selenite mixture. The component of this peak was assumed to be one of the two

compounds GSSeSG or GSSeH, mentioned previously. GSSeSG formation is favored in oxidizing conditions, low pH's, and conditions where the GSH:selenite ratio does not exceed 4:1. On the other hand, GSSeH formation is favored in alkaline pH's, reducing conditions (GSSeH is formed by the reduction of GSSeSG), and GSH:selenite ratios exceeding 4:1. Therefore, GSSeSG would probably be the predominant species in the test tube reaction of GSH/selenite as it was conducted in this study, since the GSH:selenite ratio was 1:1 and the pH was approximately 5. To have produced GSSeH would have required a GSH:SeO<sub>3</sub> ratio of greater than 4:1 and an alkaline pH (21). Therefore, the major unidentified peak in the reaction (peak 4, Fig. 8, c) is probably GSSeSG. It is also possible that peak 3 is the other logical product, GSSeH. Confirmation of the identities of these substances remains to be done.

GSSeSG also appears to be in both the in vivo and in vitro bile (Fig 8, b and c). It is conceivable that in the bile, prior to secretion, GSSeH instead of GSSeSG is the major form. This is so because the the ratio of GSH to selenite would be greater than 4:1.

Eberle et al. (22) reported that the total glutathione concentration in rat bile was approximately

4.30 mM, and that the amount of reduced glutathione (GSH) entering the bile was approximately 3.45 mM.

Since 30% of an injected dose of Se (approximately 0.175 mg Se was injected into rats weighing 0.350 kg) is eventually excreted into no more than 5 ml of bile, the GSH:Se ratio would be greater than 7:1. Also, since the pH of rat bile is basic (pH of rat bile is approximately 8.5), conditions in bile seem to favor the formation of GSSeH over GSSeSG.

## CONCLUSIONS

Selenium is an element essential for life processes, but it can also cause severe toxicity in excessive amounts. It is distributed by the circulatory system to the various organs of the body. The extent of its accumulation in the organs varies with the type of tissue, the level of selenium administered, and the individual susceptibility to selenium intoxication (59). Detoxifying organs tend to accumulate the highest quantities of selenium, and investigations on several animal species have resulted in good agreement that in both acute and chronic selenosis the highest concentrations of selenium are found in the liver and kidney.

Selenium is normally present in the protein fractions of the various organs of the body, and is known to be present as selenocysteine in the enzyme glutathione peroxidase. No other form of selenium in the proteins of mammals has been found.

When toxic levels of selenium as selenite are administered to animals, it eventually deposits throughout the body in the protein fractions causing death. However, when arsenic is administered after toxic levels of selenium, many of these animals live. Much of this selenium can be found in the bile. Thus, arsenic is believed to be an antagonist for selenium

and it is thought to exert its protection by increasing the biliary excretion of selenium. The form of selenium in bile is not known.

The objective of this work was to characterize the forms of selenium excreted in bile of rats injected with selenite followed by arsenite. On the basis of the work presented here, several conclusions can be deduced.

1) Selenium in bile is not associated with the three major bile salts; taurocholate, taurochenodeoxycholate, and taurodeoxycholate salts since the  $^{75}\text{Se}$ -activity chromatographed away from the bile salt standards during thin-layer chromatography and high performance liquid chromatography.

2) Following thin-layer chromatography of bile, two forms of selenium were observed. The main form was present at the solvent front in two systems, while the second form was located near the origin in both the toluene and chloroform systems. Thus, both lipophylic and lipophobic forms of selenium appeared to be present. However, selenium as selenite, has been found to associate nonspecifically with lipid material; therefore, the thin-layer chromatography data are consistent with the conclusion that selenium could be present in bile essentially as an inorganic form.

3) Preliminary studies (18,19) on bile showed

that the major portion of selenium in bile was associated nonspecifically with protein since the major selenium containing fraction from G-200 Sephadex gel filtration appeared at the  $V_0$  of the column. However, data presented in this study indicates that less than 10% of the radioselenium in bile was located at the  $V_0$  and the major fraction was located at the  $V_t$  of the column. These data suggest that the major portion of selenium is not associated with protein. This discrepancy can be explained by the finding that selenium adsorption to protein increased with time. A five-fold increase over one week was observed when storing bile at 4 C. Therefore, selenium probably is not associated with protein when it is transferred across the hepatocyte, but seems to be excreted in a low molecular weight form.

Other lines of evidence which also support the lack of specific incorporation of selenium in rat biliary proteins are:

- a. Much of the selenium in bile will pass through membranes when dialyzed against reduced GSH.
- b. No specific association of selenium and protein was found when proteins were fractionated into 14 distinct bands (nondenatured) or 24 distinct bands (SDS denatured) by polyacrylamide gel electrophoresis.



4) The data from G-100 Sephadex gel filtration chromatography experiments showed that selenium was not associated with the micelles. Thus, the components of the micelle including cholesterol, phospholipid, lipid, and bile salts were concluded to have no specific association with selenium.

5) Because none of the major components of bile contained selenium in a specific association, selenium was suspected to be present in bile in forms not requiring enzymatic synthesis. Several lines of evidence support this conclusion:

a. Thirty percent of the  $^{75}\text{Se}$ -activity present in the  $V_t$  of G-200 Sephadex gel filtration (90% of all radioactivity of in vivo bile was present in this fraction) will extract into cyclohexane after complexing with diaminonaphthalene. This indicates that 27% of all selenium in bile is selenite.

b. Cation and anion exchange chromatography of the same fraction indicates 20% or 40%, respectively of the  $^{75}\text{Se}$ -activity in bile is probably selenite.

c. Amino acid analysis also indicates that some selenite is secreted directly into bile. The data also indicated that the other forms of selenium present in bile can be explained by the nonenzymatic reaction of

selenite with glutathione. Proof of the identity of these substances remains to be established. Further work is also needed to establish if the effect of arsenic is directly on the synthesis of these forms of selenium or if it just increases the excretion of selenite into bile with the subsequent reaction of selenite with glutathione and its derivatives.

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